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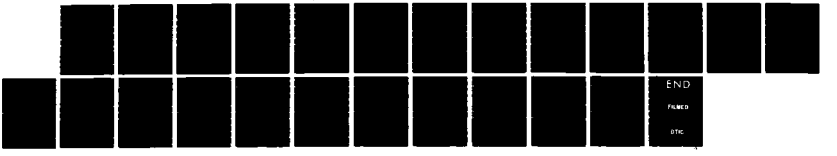

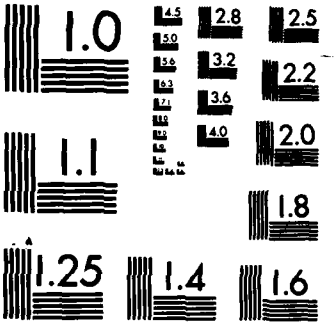

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Report AFOSR-

AFOSR-TR- 85-0629

AD-A158 974

CHEMICAL CARCINOGEN (HYDRAZINE et. al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID FIBROBLASTS IN VITRO

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410 West 12th Avenue
Columbus, Ohio 43210

12 June 1985

Final Report for Period 1 July 1980 - 30 November 1984

Approved for public release; distribution unlimited.

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Prepared for
AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Bolling Air Force Base
Washington, D.C. 20332

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

* CHEMICAL CARCINOGEN (HYDRAZINE et. al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID FIBROBLASTS IN VITRO

There is data from in vivo animal systems that DMH and polynuclear hydrocarbons may pose a potential carcinogenic risk to man. They are metabolized to reactive intermediate metabolites that are localized in suspectible target sites. These sites may be away from the tissue that activates the proximate carcinogen. One such highly reactive intermediate obtained from DMH metabolism is methylazoxy methanol. This compound purportedly degrades to form methyldimine and formaldehyde. Mehhyldiimine then forms a methyl radical after homolysis. This compound then is converted to a carbonium ion and the radical interacts with the purine bases in DNA. Methylazoxymethanol acetate, (MAMA) in the presence of -- (continued)

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colon, secum and liver homogenates reduced NAD^+ to NADH. These "alcohol dehydrogenase-"like enzymes are quite high in activity in the liver and may account for the organotypic response of MAM in animals.

We continued biochemical studies to examine how these carcinogens were activated, entered the human cell and were transported to the nculeus. We also studied how these reactive carcinogenic intermediates interacted with different bases in the DNA.

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Final Technical Report

CHEMICAL CARCINOGEN (HYDRAZINE et al.) INDUCED CARCINOGENESIS OF HUMAN DIPLOID FIBROBLASTS IN VITRO

Dates of Report: July 1, 1980 to November 30, 1984

The Ohio State University
Columbus, Ohio 43210

George E. Milo
Professor Physiological Chemistry
and Comprehensive Cancer Center
410 W. 12th Ave. Suite 302

Animal Use Statement

The experiments reported herein were conducted according to the principles described in "Guide for the Care and Use of Laboratory Animals" prepared for the Committee on Care and Use of Laboratory Animals, DHHS Publication No. NIH 78-23, revised 1982.

George E. Milo

George E. Milo, Ph.D.
Comprehensive Cancer Center

The Ohio State University has on file with the Office of Protection from Research Risks, NIH, a statement of assurance concerning the care and treatment of laboratory animals. This assurance states that the University complied with NIH Guide for the Care and Use of Laboratory Animals, applicable portions of Public Law 91-579, and related rules and regulations issued by the Secretary of Agriculture.

Richard L. Wright

Richard L. Wright
Deputy Director for Development
The Ohio State University
Research Foundation

TABLE OF CONTENTS

A final report on contract no. F49620-77-0110 was finalized in 1979. The work following that period under contract F49620-80-C-0085 was a continuation of this effort with a new work scope.

Work Scope 1980-1981

a) To investigate how UDMH, HZ and/or BP are transported to the cell nucleus.

b) To investigate changes in DNA directed DNA polymerase I and II activity following treatment with UDMH, HZ and/or BP.

c) To examine changes in histone labeling patterns during the early and late stages of the carcinogenic process following exposure to UDMH or BP.

d) To investigate the interaction of HZ and BP metabolites with DNA during the induction process and correlate this with the metabolic profiles in the activation stage.

e) To develop a predictable and reliable procedure using human cells in vitro to evaluate the carcinogenic potential of chemicals of interest to the Air Force.

Abstract

There is data from in vivo animal systems that DMH and polynuclear hydrocarbons may pose a potential carcinogenic risk to man. They are metabolized to reactive intermediate metabolites that are localized in suspectible target sites. These sites may be away from the tissue that activates the proximate carcinogen. One such highly reactive intermediate obtained from DMH metabolism is methylazoxy methanol. This compound purportedly degrades to form methyldiimine and formaldehyde. Methyldiimine then forms a methyl radical after homolysis. This compound then is converted to a carbonium ion and the radical interacts with the purine bases in DNA. Methylazoxymethanol acetate, (MAMA) in the presence of colon, secum and liver homogenates reduced NAD^+ to NADH. These "alcohol dehydrogenase-"-like enzymes are quite high in activity in the liver and may account for the organotypic response of MAM in animals.

We completed biochemical studies to examine how these carcinogens were activated, entered the human cell and were transported to the nucleus. We also studied how these reactive carcinogenic intermediates interacted with different bases in the DNA.

Conclusions: 1980-1981

1. BP was transported to the nucleus via a lipoprotein complex where it was activated to an oxygenated form. The 7R-BPDEI-(+) anti form of BP interacted with guanine to form the proper adduct to induce a carcinogenic response.

2. The hydrazine compounds, 1,1- and 1,2-dimethyl forms of HZ alkylated directly the DNA, namely, N^7 of guanine and O^6-guanine. Again using a compound not requiring activation the appropriate DNA-adducts were formed.

3. The metabolic profiles of BP metabolism by the plasma membrane associated P_{450} oxygenase enzymes indicated that this activation process was primarily associated with producing toxic metabolites that resulted in cellular toxicity rather that a carcinogenic respose.

4. There were no changes in α, β or DNA polymerase activity of cells treated with a carcinogenic non-toxic dose of BP or 1,1-; 1,2-DMH. Furthermore, semi-conservative DNA synthesis pattern of synchronous cells in S was not altered. The profile of S was determined by autoradiography following the incorporation of [^{3}H-CH_3] -thymidine into DNA.

5. We detected no change in methylation, acetylation or phosphorylation of histone proteins as reported for rodent cell systems at the time of initiation. At the time of expression of the transformed phenotype 10 PDL following the conclusion of treatment, we did at that time see a change in labeling patterns of histones primarily in the H_1 histone.

6. We found that FeSV transformation and U.V.$_{254}$ nm induction of transformation followed a similar pattern of induction of transformation, i.e. in a narrow window of time in early S the cells were optimally responsive to the carcinogenic insult.

Papers Published 1980-1981

1. Milo, G., R. Olsen, S. Weisbrode, and J. McCloskey (1980) Feline sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells. In Vitro. 16: 813-822.

2. Milo, G., and J. DiPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Int. J. Cancer. 26: 805-812.

3. Milo, G., A. Aackerman, and I. Noyes (1980) Growth and ultrastructural characterization of proliferating keratinocytes in vitro without added extrinsic factors. In Vitro 16: 20-30.

4. Tejwani, R., S. Nesnow, and G. Milo (1980) Analysis of intracellular distribution and binding of benzo(a)pyrene in human diploid fibroblasts. Cancer Letters 10: 57-65.

5. Milo, G., and J. DiPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Int. J. of Cancer. 26: 805-812.

6. Milo, G., S. Weisbrode, R. Zimmerman, and J. McCloskey (1981) Ultraviolet radiation induced neoplastic transformation of normal human cells. Chem. Biol. Int.36: 45-59.

7. Milo, G., R. Trewyn, R. Tejwani, and J. Oldham (1981) Intertissue variation in benzo(a)pyrene metabolism by human skin, lung and liver, in vitro. Advisory group for aerospace research and development. Sci. Tech. Aerospace Rep. 18: 2533-2541.

Work Scope 1981-1982

a) To examine changes in histone labeling patterns or carcinogen-nuclear non-histone protein binding during the early and late stages of the carcinogenic process following exposure to different carcinogens.

b) To investigate the interaction of BP and/or BP metabolites with DNA during the induction process and correlate these adducts with BP oxygenated metabolites produced during the activation stage.

c) To investigate different nitrosamines for their carcinogenic activities.

d) To investigate the role of promoters (modulators) in the induction process.

e) To study the interaction of BP and/or BPDE-I with cellular DNA of responsive and refractory cell populations.

f) To investigate the role of the cell cycle in influencing chemical induction of human cell transformation.

g) To investigate the effects of hydrazines and their analogues on unscheduled DNA synthesis.

Abstract

Polynuclear hydrocarbons or hydrazine analogues do not have an absolute requirement for binding to DNA in order to elicit a carcinogenic event. Furthermore, PNH like BP enter the nucleus via a lipoprotein complex where the BP is oxygenated and then interacts with the genetic material, (DNA). The adduct formed is 7&-BPDEI-dG. Benzamide does not alter the binding of the perceived ultimate carcinogen to dG but eliminates the carcinogenic response. The period of heightened response to the carcinogen appears to be 3 hrs into S phase of the cell type. The modifying effect of TPA or benzamide appears to alter the nuclear non-histone proteins protecting the cellular DNA.

Conclusion: 1981-1982

The specific points addressed in the previous years' conclusions were then examined again in the presence of benzamide (BZ), an inhibitor of the carcinogenesis process.

1. It was found that the BZ compound, while inhibiting the expression of carcinogenesis, did not alter the profile of specific carcinogen-DNA adduct formation.

2. Unscheduled DNA synthesis in the presence of and/or absence of BZ was not altered. Moreover, when an insult was delivered to the cells in G_1 part of the cell cycle compared to S there was no difference in repair of the lesions in DNA. However, cells treated in G_1 exhibited no carcinogenic response to the insult while cells treated in S did exhibit a carcinogenic response.

3. Human foreskin fibroblast populations blocked in G_1, released and treated with methylazoxy methanol acetate (MAMA) from the time of release (late G_1) for 1 hr treatment intervals until 4 hrs into S, exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell regulatory protein, was optimally present in the nuclei of the late G_1 treated cells 6 hrs after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes, (cells that will grow in soft agar) observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 hrs after release from the block 2-3 hrs into S. Interestingly, this was followed by a decrease in the expression of anchorage independent growth when the cells were treated 13-14 hrs after release from the block 4 hrs into S. Benzamide interfered in the process when added at the onset of S and the resultant carcinogen treated population did not exhibit a comparable increase in expression of anchorage independent growth. Cells treated with MAMA at the point of release from the block $G_1{}^{0-1}$ to $G_1{}^{3-4}$ did not express anchorage independent growth.

4. Cell cycle studies to study the effects of phorbol esters on cell cycle activation using the published cytofluorometric procedures developed for animal cell systems were not translatable to human cells. Dr. D. Tomei had to abandon the published techniques designed for animal studies and reevaluate the application of published systems to human cell kinetic cell cycle work. The vital stains used to stain cellular DNA would not work. We are continuing to evaluate these stains as described above. Either scheduled DNA synthesis in human cells undergoes a multiple initiation point of the beginning of DNA synthesis or the intercalating DNA dyes do not penetrate the nucleus in the same manner as that described for animal cells. By modifying our isolation procedures we now are obtaining yields in excess of 50% clean nuclei. The application of these techniques permitted us to investigate the toxic effects of compounds on the nuclei of the layers of skin grown in vitro.

Papers Published 1981-1982

1. Tejwani, R., R. Trewyn, and G. Milo. (1981) Kinetics of movement of benzo(a)pyrene into transformable and nontransformable diploid fibroblasts. Symposium on Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, Battelle Mem. Inst. 5:97-107.

2. Cazer, F., M. Inbasekaran, J. Loper, R. Tejwani, D. Witiak and G. Milo (1981). Human cell neoplastic transformation with benzo(a)pyrene and a bay region reduced analogue of 7,12-dimethylbenz(a)anthracene. Symposium on Polynuclear Aromatic Hydrocarbons, Fifth International Symposium, Battelle Mem. Inst. 5:499-507.

3. Milo, G., J. Oldham, R. Zimmerman, G. Hatch, and S. Weisbrode (1981) Characterization of human cells transformed by chemical and physical carcinogens in vitro. In Vitro. 17: 719-729.

4. Donahoe, J., I. Noyes, G. Milo, and S. Weisbrode (1982) A comparison of expression of neoplastic potential of carcinogen-transformed human fibroblasts in nude mice and chick embryonic skin. In Vitro. 18: 429-434.

5. Tejwani, R., A Jeffrey, and G. Milo (1982) Benzo(a)pyrene diol epoxide DNA adduct formation in transformable and nontransformable human foreskin fibroblast cells in vitro. Carcinogenesis. 3:727-732.

6. Milo, G., and R. Trewyn (1982) In vitro transformation of cultured human diploid fibroblasts. Banbury Report. Nitrosamines and Human Cancer. Edited by Peter N. Magee. Cold Spring Harbor Laboratory. 12: 3-13.

Work Scope 1982-November 30,1983

a) To develop a procedure that is both predictable and reliable, using human cells in vitro to evaluate the potential carcinogenic activity of suspect carcinogens.

b) To study the effect promoters and sensitizers have on the preinduction process.

c) To study the interrelationships between analogues of carcinogens and their carcinogen activity.

d) To investigate the role of the cell cycle in influencing chemical induction of human cell transformation.

e) To continue studies directed towards understanding the role of the carcinogens evaluated in 1981-1982 scope of work.

Summary

We have developed a reliable and reproducible biological endpoint to evaluate the carcinogenic endpoint that extends beyond the work published in IN VITRO 17: 719-729, 1981. We can evaluate the endpoint for an expression of cellular invasiveness on chick embryonic skin in vitro. We can evaluate the neoplastic potential of the transformed cells upon injection of a bolus of transformed human cells intracranially into the frontal sinus of a nude mouse. We can reliably and reproducibly evaluate the transformed cells for the presence of a sarcoma associated determinant using a monoclonal antibody directed against the cell surface determinant. We have tissue typed a range of sarcoma tumor tissues from humans and found one case that was negative in $ 25 tumors evaluated to date.

Secondly, we have examined each phase of the cell cycle for its responsiveness to a carcinogenic insult. Human foreskin fibroblast populations blocked in G_1, released, and treated with methylazoxymethanol acetate (MAMA) from the time of release (late G_1) for 1 hr treatment intervals until 4 hrs into S exhibited a differential sensitivity to MAMA treatment at the different treatment times. A heightened response to the carcinogen treatment was not detected until calmodulin, a cell-regulatory protein, was optimally present in the nuclei of the late G_1-treated cells 6 hrs after release from the G_1 block. Moreover, there was a distinct increase in the number of transformed phenotypes (cells that will grow in soft agar), observed when the cells were treated with MAMA at the onset of scheduled DNA synthesis. The time at which these treated cells were optimally responsive to a carcinogenic insult was 12-13 hrs after release from the block 2-3

hrs into S. Interestingly, this was followed by a decrease in the expression of anchorage-independent growth when the cells were treated 13-14 hrs after release from the block 4 hrs into S. Benzamide interfered in the process when added at the onset of S, and the resultant carcinogen-treated population did not exhibit a comparable increase in expression of anchorage-independent growth. Cells treated with MAMA at the point of release from the block G_1^{0-1} to G_1^{3-4} did not express anchorage-independent growth.

It was proposed that the heightened presence of calmodulin in the nuclei 4 hrs prior to the onset of scheduled DNA synthesis is a cell-regulatory function that sets in motion a complex series of events (program) in carcinogen-initiated human fibroblasts that leads to a subsequent carcinogenic response.

The specific DNA-carcinogen adducts formed under these conditions were qualitatively and quantitatively no different in non BZ-carcinogen treated cells when compared to BZ-carcinogen treated cells. We conclude from these studies that specific adduct formation between the carcinogen and bases in the DNA does not appear to be the critical mechanism for induction of a transformed human phenotype. We recognized that the toxic insult plays a role in the program of cellular events that leads to a carcinogenic response but is not necessarily a requirement when the events leading to an initial expression of carcinogenesis are not properly programmed into the dynamic cell cycle phase.

In the narrow window of time where the cell in S is responsive to the carcinogenic insult (Milo, G. (1983) In vitro transformation of human cells: modulation of early gene expression preceding carcinogen induced events. Human Carcinogenesis. Eds. Harris and Autrup, Chapt. 17: 431-449. Academic Press, N.Y.) we do not know whether the carcinogen-adducts formed in early S are critically formed in the fast replicating DNA or the parental strand of DNA. We are at the present engaged in the following experiments to evaluate the above:

1. Experiments to look at specific carcinogen-DNA adducts in fast replicating and parental DNA in the presence of BZ.

2. Using either [^{3}H]-G or [^{14}C]-G labeled Budr we will examine the isolated Budr labled DNA in the light and heavy strands of DNA to see if there is preferential binding of the carcinogen in the presence or absence of BZ.

3. We are developing post $^{32}P_i$ labeling technology to measure carcinogen-DNA adducts at the fentamole (10^{-15}moles) level rather than the micromole (10^{-6}moles) level. This technology will permit us to increase our level of sensitivity for detection of specific carcinogen minor modification of the DNA and quantitate minor modifications in the DNA. The addition of BZ to the carcinogen treated cultures will permit us to examine how the cells will respond to a toxic insult. Another biological response the treated cells may express is a mutagenic response. Under these conditions, when grown on 6-thioguanine supplemented medium, the cells produce mutants. These hprt mutants were amplified when the cells were treated in late G_1 4 hrs prior to the onset of S. In the presence of BZ, added at the G_1 release point for 10 hrs and removed at the onset of S, transformants were reduced to zero while not affecting the expression of the point mutations, (hprt mutants). We can, therefore, by selecting when we add the carcinogen, either elicit a mutagenic response, a carcinogenic response, or a toxic response. We suspect, due to the short period of time that elapses between the initial insult and the fixation of the insult that the initial insult is fixed in the cell in the following manner:

We will study these proposed pathways by examining the treated cells for deaminase activity, incorporation of radiolabeled methionine into BZ and non-BZ treated cells, etc.

Publications 1982-1983

The work on the inhibitors of the carcinogenesis process in collaboration with Dr. E. Kun was forwarded to AFOSR last fall in an annual report. This work was not reported on under this cover. Furthermore, the collaborative work with Dr. Donald Witiak is presently being written into three manuscripts, one which is included under this cover, and two in preparation.

1. Howard, P., J. Gerard, G. Milo, P. Fu, F. Beland and F. Kadlubar (1983) Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo(a)pyrene. Carcinognensis 4: 353-355.

2. Milo, G. (1983) In vitro transformation of human cells: modulation of early gene expresison preceding carcinogen induced events. Human Carcinogenesis. Edited by Curtis Harris and Herman Autrup. Chapt. 17: 431-449. Academic Press, N.Y.

3. Kun, E., E. Kirsten, G. Milo, P. Kurian, L. Kumari (1983) Cell cycle-dependent intervention by benzamide of carcinogen-induced neoplastic transformation and in vitro poly (ADP ribosyl)ation of nuclear protein in human fibroblasts. Proc. Nat. Acad. Sci. 80:7219-7223.

4. Kun, E., T. Miraga, E. Kirsten, G. Jackowski, J. McLick, L. Peller, S. Oredsson, L. Martin, N. Pattabiraman, and G. Milo (1983) Biochemical basis of the regulatory role of polyadenosine diphosphoribose. Adv. in Enz. Reg. 21: 177-197.

5. Cazer, F., S. Barnela, K. Kumar, P. Kumat, G. Milo and D. Witiak (1983) Synthesis of ^{14}C-labeled methylazoxy methanol acetate of high specific activity. J. of Labeled Compds. and Radiopharmaceuticals. In press.

6. Kumari, L., P. Kamat, S. D'Ambrosio, D. Witiak, and G. Milo (1983) DNA modification and damage by dimethylhydrazine and methylazoxymethanol acetate. Neoplastic transformation and cytotoxicity of human diploid cells. Cancer Res. in preparation.

7. Kumari, L., D. Witiak and G. Milo (1983) Effect of anticarcinogen, benzamide on molecular perturbation of DNA by MAMA. Transformation of human cells. Carcinogenesis in preparation.

WORK SCOPE FINAL YEAR 1983 - 1984

On Jan 3, 1984 a letter was sent to Lt. Col. Lind, Program Manager Life Sciences Directorate, Department of the Air Force outlining the work scope for 1984.

Work Scope 1984

a) Using human skin we will transplant the normal skin onto nude mice, treat the skin grafts with toxicants and study
   a. Carcinogen-DNA adduct formation
   b. Presence of transformed phenotype
   c. Metabolite profile to the toxicant

b) The other area(s) of interest are studies we have initiated to examine the effects of inhibitors on the expression of the transformed phenotype following the administration of inhibitors to transfected DNA chemically transformed cells.

c) Lastly, it appears that the initial stage of expression of a malignant phenotype, once a lesion in the DNA has occured, may be by incorporation of a miscoded base into the DNA. We hypothesize this may occur in the following manner:

> We are going to test this proposal by looking for the incorporation of [^{3}H-CH_3]-methionine into cytosine and then using restriction enzymes, cut the DNA into segments and examine the segments of DNA for the presence of methylated cytosine and after 4 hrs look for Me-Uracil bases by HPLC analysis of the Me-Uracil nucleotide by post $^{32}P_i$ labeling technique.

Summary

Objective a. (1984) we have successfully transplanted human skin onto a nude mouse. The xenograft remains in place for more than 24 weeks. This was accomplished by further immunosupressing the immunoincompetent surrogate host. Upon completion of the identification of carcinogen-DNA adducts procedure, (To be submitted to Carcinogenesis in 1985), we ran 2 experiments on the human skin xenograft to investigate the benzopyrene[a] diol-epoxide I modification of the replicating DNA compared to the parental DNA with BPDE-I. We have found that we can determine the extent of modification of nuclear DNA in human cells down to the fentamole level, i.e. 10^{-15} moles of adduct. (These data will be presented at the National Meeting of the Society of Investigative Dermatology in 1986.) Once the critical adducts are formed the challenge was to detect the presence of a transformed phenotype. Included here is an abstract of a publication accepted by Cancer Research for publication in 1985.

"A comparison was made of the in vitro characteristics of tumor cells versus chemically transformed fibroblasts. We focuses on the characteristics of the malignant tumor phenotype as a basis to compare the behavior of and progressive development of the carcinogen transformed cells. To date, only one sarcoma line out of 50 evaluated, exhibited an infinite lifespan. The range of population doublings observed for the remaining 49 lines was from 5 to 174, an extended, but finite lifespan. All of these cells reacted with the anti 115 KGP monoclonal

antibody. Immunohistochemical staining of frozen sectioned surgically removed sarcoma lesions detected no reativity of the MoAb 345.134S with the surrounding normal tissue. This monoclonal antibody reacted with all carcinogen transformed phenotypes that exhibited anchorage independent growth following their growth in soft-agar. Transformed cells that exhibited altered morphology before anchorage independent growth did not react with the monoclonal antibody. There appeared to be a requirement for time in culture for the progressive development of the altered phenotype that took ca. 16 population doublings under the selection pressure used. Both populations from the tumor tissues and the carcinogen transformed phenotypes formed only localized tumors when injected subcutaneously into the subscapular area of a nude mouse. If inoculation was by intracranial route, cells proliferated and formed a tumor mass that created neurological dysfunction followed by death of the surrogate host. Both cell type exhibited cellular invasiveness when inoculated onto 9 day old chick embryonic skin in vitro."

The metabolite profile of the Benzo[a] pyrene diol-epoxide I administered to the xenograft on the surrogate host have not been completed as of this date.

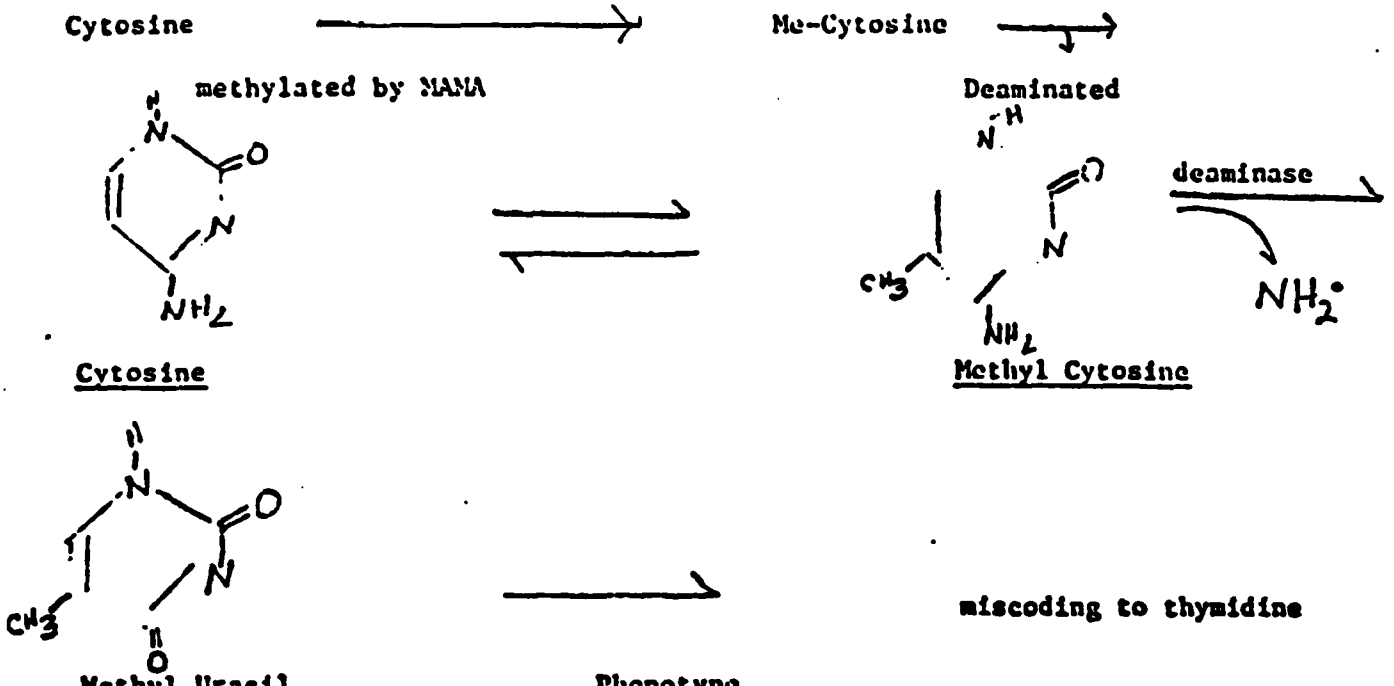
The other two objectives are presently under investigation and the final data will be forthcoming. The technology has been worked out to complete the last two objectives and as soon as we complete gathering the data we will send the publication to the agency.

Publications 1984

1. Milo, T.E., Kurian, P., Kirsten, E. and E. Kun (1985) Inhibition of carcinogen-induced cellular transformation of human fibroblasts by drugs that interact with the poly(ADP-ribose)polymerase system. FEBS 179:332-336;

FIGURE 1

Genetic Coding Mechanism



Phenotype

C≡ C

↓ methylation during S

C≡ C-Me

↓

C≡ G Uracil-Me replicating DNA

parental DNA ↳ daughter cell Uracil codes with T

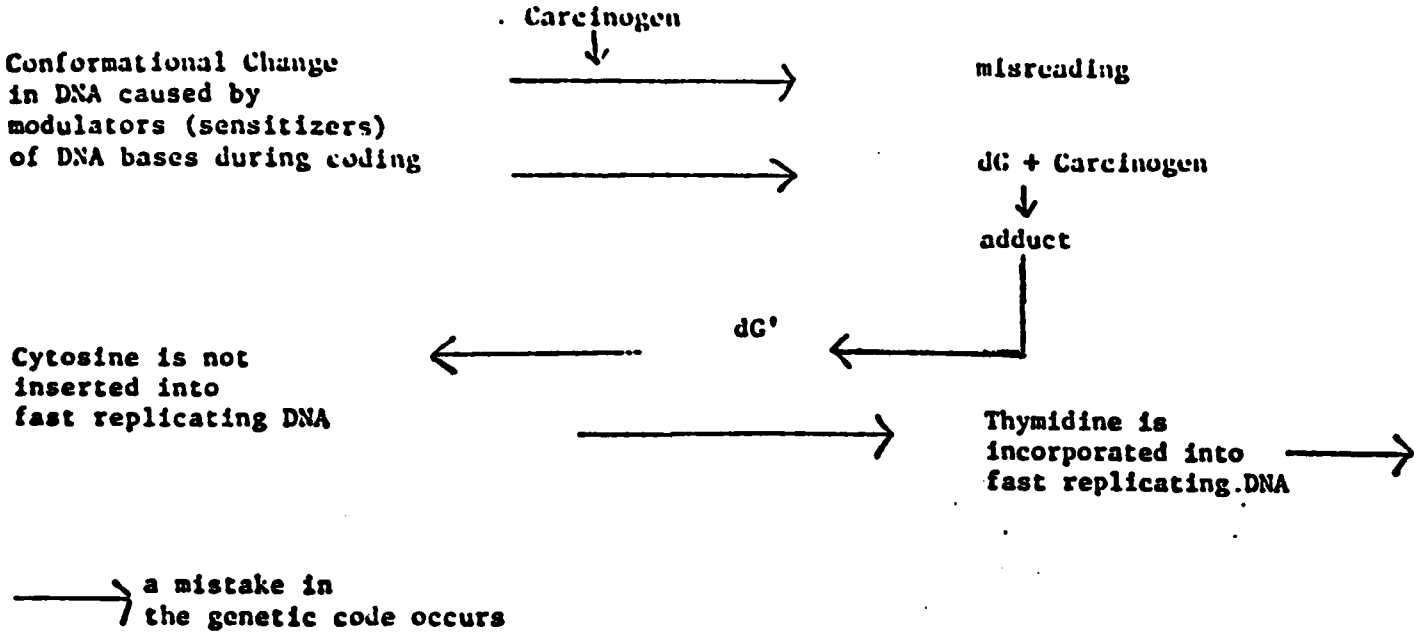
↙

Reading frame shifts

FIGURE 2

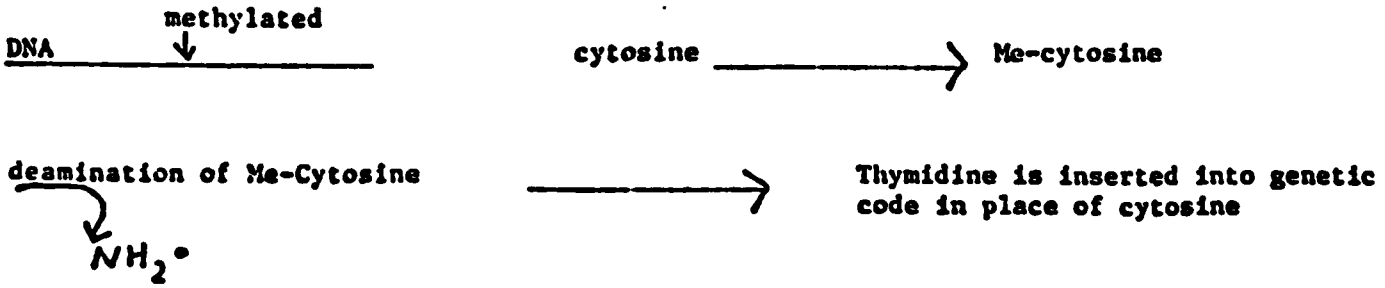
Proposal: Molecular Mechanisms to Explain Genotoxic Insults

1. Bulky Adducts



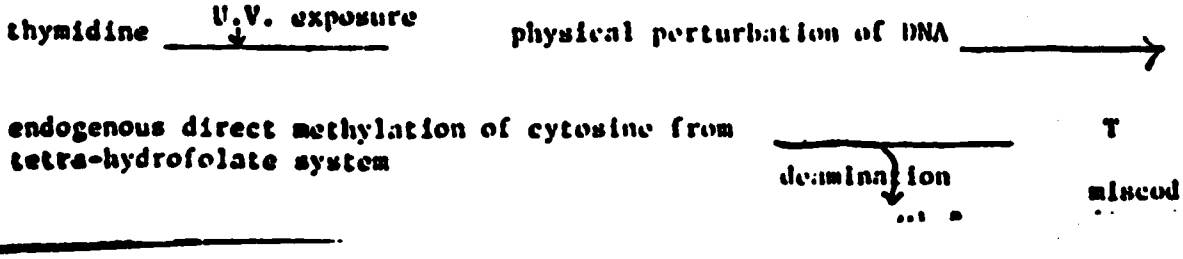
2. Non-bulky adducts

These are formed by akylating agents such as 1,1- or 1,2-UDMH



3. Physical Carcinogen

U.V. transformation



Inhibition of carcinogen-induced cellular transformation of human fibroblasts by drugs that interact with the poly(ADP-ribose) polymerase system

Initial evidence for the development of transformation resistance

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Two types of interactions of 13 drugs with human fibroblasts were determined: (a) I_{50} of nuclear poly(ADP-ribose) polymerase, as assayed with isolated nuclei in vitro, and (b) the non-toxic concentration of drugs that prevented carcinogen-induced cell transformation of intact fibroblasts (RCF_1). In general, RCF_1 was much lower than I_{50}, and one antitransformer did not inhibit the enzyme in vitro, indicating that low-affinity enzyme inhibitory sites appear to play no role in the mechanism of prevention of cell transformation. Two enzyme inhibitors, caffeine and 1-methylnicotinamide, exhibited no antitransforming activity. Benzamide when applied in population doubling 1 induced resistance to cell transformation in population doubling 6 by carcinogens added at this stage.

Human fibroblast *Cell transformation* *Poly(ADP-ribose)polymerase*

1. INTRODUCTION

An inhibitor of poly(ADP-ribose) polymerase, benzamide [1], when present at non-toxic concentrations together with equally non-toxic quantities of ultimate carcinogens, prevents the induction of cell transformation in human fibroblasts that takes place in the absence of benzamide [2]. Similar observations were reported with C3H10T½ hamster embryo cells [3]. The antitransforming action of benzamide is confined to the early S phase and coincides with an increase of poly(ADP-ribose) polymerase activity [2], similar to the apparent induction of this enzyme in liver nuclei following benzamide feeding [4]. It would be expected that the multi-stage process of cellular transformation, leading to neoplasia, might be arrested by various agents at various stages (e.g., by prolonged exposure to protease inhibitors, cf. [5]) and the observed prevention of transformation by non-toxic concentrations of certain molecules that bind to the poly(ADP-ribose) polymerase system [2,3] is likely to indicate specific sites probably related to initiation. We show here that several in vitro inhibitors of poly(ADP-ribose) polymerase, at concentrations much below I_{50}, can function as antitransformers in intact cells. Conversely, some agents known to prevent carcinogenesis in animals can also inhibit poly(ADP-ribose) polymerase in vitro at relatively high concentrations. Therefore, the antitransforming propensity of molecules cannot be fully correlated with an inhibition of poly(ADP-ribose) polymerase and the cell biological effect of antitransformers is more probably reflected in high-affinity nuclear binding sites (RCF_1 as compared to I_{50}).

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2. EXPERIMENTAL

Preparation, culturing, synchronization, the method of exposure of human fibroblasts to carcinogens and to antitransforming drugs, and the assay technique for poly(ADP-ribose) polymerase, following quantitative removal of adsorbed drugs from the cell surface by both trypsin and antitrypsin treatment, have been reported [2,7]. Ultimate carcinogens employed were methylazoxymethanol acetate (MAMA, 7 μM) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, 0.7 μM) which were used interchangeably because at these non-toxic doses their transforming effects were indistinguishable. It is important to note that these low non-toxic concentrations of carcinogens are far below the doses many investigators use routinely in cell cultures to achieve responses in rates of poly ADP-ribosylations. For example, in a recent representative report MNNG was employed at nearly 50-times higher concentrations (cf. [8]) than in our studies. Toxicity, or rather its absence, was monitored by comparing cloning efficiencies in the presence and absence of drugs [2] and drug concentrations defined as RCF_1 (where relative cloning frequency is equal to one, meaning that cloning efficiencies with and without drugs are identical) were used. This criterion, as illustrated in fig.1 for benzamide, has been strictly employed for all drugs and drug combinations [2]. After exposure of cells in S of PD_1 (population doubling 1) to drugs, passages without drugs were continued for 20 PD and transformation frequencies were then determined by colony counts of transformed cells after transfer of cultures to a soft (0.33%) agar medium, representing anchorage-independent growth [2,7]. The dose response between the concentration of a typical antitransformer and the decrease in the number of colonies in soft agar (fig.1) portrays a sensitive quantitative measure of inhibition of cell transformation. For comparison of many drugs (table 1) only one concentration of antitransformer drug (i.e., RCF_1) is given, instead of a dose response curve which would involve almost unmanageable numbers of culture plates. For a total of 82 experimental series (table 1) between 1.6 and 2.0 $\times$ 10^6 transformed colonies were counted (one colony = a minimum of 50 cells) and the average rate of transformation by carcinogens was between 40 and 50 colonies per 10^4 cells [2].

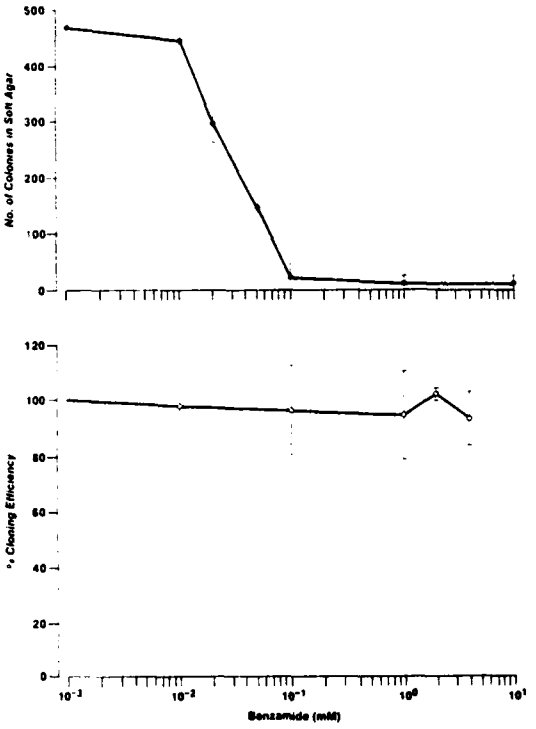

Fig.1. Correlation between the concentration of extracellular applied benzamide (between 0 and 1 mM, abscissa) and the number of transformed cell colonies capable of growing in soft agar (expressed as number of colonies/100000 cells; ordinate of upper curve). The transforming agent was 7 μM methylazoxymethanol acetate. The lower curve indicates the absence of cellular toxicity of either 7 μM methylazoxymethanol acetate (not shown separately) and varying concentrations of benzamide alone or in combination with the carcinogen. Ordinate of lower curve is % cloning frequency; eight parallel experiments were performed according to [2]: error bars represent SD, n = 8. RCF_1 is by definition between 0.1 and 1 mM benzamide.

Enzyme inhibitory indices (I_{50}) and the nature of inhibition were determined by standard kinetic models [9].

3. RESULTS AND DISCUSSION

As summarized in table 1, 10 out of 13 substances that at millimolar concentrations inhibit in vitro the nuclear poly(ADP-ribose) polymerase system, as determined by initial veloci-

Table 1

Prevention of carcinogen-induced cellular transformation of human fibroblasts by drugs that are inhibitory on *poly(ADP-ribose) polymerase in vitro*

No.	Experimental conditions	No. of transformed colonies per 5×10^4 cells	No. of experimental series	I_{50} (M)
1 (a)	0.7 μM MNNG or 7 μM MAMA	244.28 ± 13.6	21	–
(b)	a. + 1 mM BA	0	2	0.5×10^{-3}
(c)	1 mM BA	0	2	
2 (a)	+ 0.1 mM BHA	1.4 ± 0.6	2	
(c)	0.1 mM BHA	1.3 ± 0.3	2	5.0×10^{-3}
3 (a)	+ 0.1 mM Me-BHA	1.0 ± 0.4	3	
(c)	0.1 mM Me-BHA	0	3	20.0×10^{-3}
4 (a)	+ 0.7 μM NAL	2.0 ± 1.5	2	
(c)	0.7 μM NAL	0	2	2.0×10^{-3}
5 (a)	+ 0.8 μM NOV	8.0 ± 2.3	2	
(c)	0.8 μM NOV	0	2	10^{-3}
6 (a)	+ 0.2 μM LEV	6.0 ± 1.8	5	
(c)	0.2 μM LEV	0	5	10^{-3}
7 (a)	+ 69 μM COU	1.0 ± 0.7	5	
(c)	69 μM COU	13.0 ± 4	5	10^{-3}
8 (a)	+ 3 μM QU	2.8 ± 0.8	2	
(c)	3 μM QU	27.0 ± 3.3	2	0.25×10^{-3}
9 (a)	+ 0.4 μM ISO	0	2	
(c)	0.4 μM ISO	0	2	0.4×10^{-3}
10 (a)	+ 1 mM 1-Me-NA	220.0 ± 8.0	2 cf. [6]	
(c)	1 mM 1-Me-NA	2.0 ± 1.3	2	
11 (a)	+ 1 mM HMBA	4.0 ± 1.3	2	
(c)	1 mM HMBA	0	2 not inhibitory at 20 mM	
12 (a)	+ 1 mM CAFF	210.0 ± 15.0	2 cf. [6]	
(c)	1 mM CAFF	0	2	
13 (a)	0.8 μM PRIM	4.0 ± 1.5	2	2.0×10^{-3}
(c)	0.8 μM PRIM	10.0 ± 8.0	2	

BA, benzamide; BHA, butylated hydroxyanisole; Me-BHA, methyl ether of BHA; NAL, nalidixic acid; NOV, novobiocin; LEV, levimasole; COU, coumarin; QU, quercetin; ISO, isoquinoline; 1-Me-NA, 1-methylnicotinamide; HMBA, hexamethylene bisacetamide; CAFF, caffeine; PRIM, primycin. The cell biological effects of selected molecules were determined in synchronized human fibroblasts exactly as in [2]. Briefly, G_1 block was produced in freshly isolated and subcultured human fibroblasts (5×10^3 cell/cm^2) by nutritional deprivation (cf. [2]) then S induced by refeeding + insulin. Transforming agents and drugs were added in early S phase. The window of effectivity of both agents was the same as described (cf. [2]). Passages for 20 population doubling were continued and treated and control cultures seeded ($3–20 \times 10^6$ cells) into a semi-solid medium (0.33% agar, cf. [2]) to score for anchorage-independent colony growth (1 colony is defined as a minimum of 50 cells). The drug concentrations given in section 2 are RCF_1 (i.e., a non-toxic dose that inhibits transformation by 85–95%)

ty enzyme kinetics [9], proved to be potent antitransforming drugs. With the apparent exception of benzamide the concentrations of drugs sufficient to prevent nearly completely transformation are far below I_{50}. The anomalous behavior of benzamide is explained by its poor cellular penetration

(less than 1% of externally added drug appears in the nucleus, cf. [2]). Caffeine (no.12) and 1-methylnicotinamide (no.10) which are known inhibitors of the enzyme (cf. [6]) do not act as antitransformers, therefore in vitro kinetic effects alone are insufficient to predict an antitransforming property. The process leading to transformation inhibition takes about 10 h [2] whereas inhibition kinetics is determined within 1–2 min. It follows that differences in drug concentrations that cause enzyme inhibition or prevention of transformation suggest the participation of at least two types of binding sites and only the high-affinity site (RCF_1) is relevant to the prevention of transformation. Recent evidence shows the participation of a second nuclear binding site for benzamide which is localized at the coenzymic DNA of poly(ADP-ribose) polymerase (in preparation; and Proceedings of the VIIth International Symposium on ADP-ribosylations, Vitznau, Switzerland, Sept. 23–27, 1984) and we presume that other antitransformers also bind to this site. This question is the subject of further studies.

Benzamide (no.1) and its *ortho*- and *meta*-fluoro analogs, *m*-methoxybenzamide and the 2-(acetonyloxy),-2-(phenylacetonyloxy)-5-chlorobenzamides (not shown) are comparable antitransformers. Benzamides are toxic at 5 mM, except for the acetonyloxychloro derivatives which are nontoxic even at saturation. Butylated hydroxyanisole (no.2), a known inhibitor of carcinogenesis in animals [10] and its *o*-methyl derivative (no.3), prevent transformation in the cell culture system. The antibiotics, nalidixic acid (no.4) and novobiocin (no.5), at millimolar concentration, exert an inhibitory effect on eukaryotic topoisomerases [13,14] as well as on poly(ADP-ribose) polymerase (table 1), suggesting DNA-related binding sites. However, the antitransforming effect of these antibiotics (nos 4,5) and of the antibiotic primycin (no.13) occurs at much lower concentration than I_{50}, similar to levimasole, a drug (no.6) that is known to be supportive in cancer chemotherapy [11,12]. The bioflavonoid derivatives quercetin and coumarin (nos 7,8) are potent antitransformers at 3 and 69 μM, respectively. Quercetin has been shown to suppress tumor promotion [15] and contrary to previous reports is not a carcinogen [16], thus the marginal induction of transformed colonies by quercetin alone, that is abolished by the simultaneous presence of a potent carcinogen, may have little biological importance. The inhibitory effect of quercetin on tyrosine phosphokinase [17] and on other enzymes of the plasma membrane [18] requires 50–100-fold higher concentrations than sufficient for the prevention of carcinogen-induced phenotypic transformation (table 1, nos 7,8). Therefore, it seems improbable that these enzymes are significantly affected by quercetin when prevention of cell transformation takes place. Isoquinoline (no.9) bears structural homology to benzamide with respect to the position of the N atom relative to the benzene moiety.

Hexamethylene bisacetamide (no.11) is known to induce differentiation in erythroleukemia cells [19] simultaneously with an increase of poly(ADP-ribose) polymerase activity [20]. Its antitransforming effect in human fibroblasts suggests that the phenomenon of induced differentiation [19] and of induced resistance to transformation, both coinciding with an increase of poly ADP-ribosylation, may be related. Hexamethylene bisacetamide at 1 mM external concentration (RCF_1) almost completely prevented carcinogen-induced transformation, but even at 20 mM had no appreciable inhibitory effect on nuclear poly(ADP-ribose) polymerase. In the past [2] and in the majority of present experiments, prevention of transformation was demonstrated when both carcinogens and antitransforming drugs were present simultaneously

Table 2

Induction of resistance to cell transformation by benzamide

No.	Experimental conditions	No.of transformed colonies per 10^5 cells after PD_{20}
1	PD_1 0.7 μM MNNG alone	185 ± 18 (2)[a]
2	1. + 1 mM BA	5 ± 2 (2)
3	1 mM BA alone	0 (2)
4	1 mM BA at PD_1 and 0.7 μM MNNG at PD_6	0 (2)
5	0.7 μM MNNG alone at PD_6	130 ± 18 (2)

PD_1 and PD_6, population doubling 1 and 6, respectively; (2)[a], 2 experimental series; the cells used in these experiments exhibited greater resistance than the cultures shown in table 1, indicating biological variations. BA, benzamide

in early S phase. However, one exposure to benzamide alone at PD_1 confers resistance to transformation as demonstrated by the ineffectivity of the subsequent addition of carcinogens at PD_6, when traces of benzamide have long been removed by 6 serial passages involving exchanges of culture media (table 2). It is probable that benzamide is not unique in producing resistance to transformation. The mechanism of this phenomenon is subject to further studies. The antitransforming effect of certain drugs (table 1) depends strictly on their low concentration that must be at non-toxic levels. Raising the drug concentration to I_{50} will not only abolish the antitransforming effect but also produce cell toxicity and can reinforce carcinogens (cf. [2]).

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